

Peer Review File

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Reviewer A

The manuscript in title of Activation of the iNOS/NO/cGMP pathway by Revactin® in Human Corporal Smooth Muscle Cells aims to explore whether a commercial formulation of COMP-4, Revactin®, could stimulate the production of inducible nitric oxide synthase (iNOS), nitric oxide (NO), and cGMP in cavernosal smooth muscle cells (CSMC) in vitro. This is very interesting project and well been conducted. The manuscript is also well prepared. However, there are several items should be fixed before it could be accepted for publication.

Comment 1. The COMP-4 is a mixture of four components, including ginger, Paullinia cupana, muira puama and L-citrulline. How those molecules bind to the smooth muscle cells or enter the smooth muscle cells to take biological effects? This should be well discussed and addressed.

Reply 1: *HPLC/spectrometric analyses of muira puama, Paullinia cupana, and ginger rhizome have been published and demonstrate that the presumed active components within these 3 nutraceuticals, as a result of their aromatic composition, can enter the cells by diffusion. For the fourth component of COMP-4 (Revactin®), L-citrulline, we have demonstrated the presence of the amino acid transporter SLC38A1/NAT2 in HCSMC that allows L-citrulline to enter the cells (new figure #6). There was no change in the expression of SLC38A1/NAT2 with L-Cit or Revactin®.*

Changes in the text, *We have added a new figure 6 and the description of the figure is included in the results section, page14, lines 13-17.*

Comment 2. The author applied a very high concentration in cell culture, it was 345ug/ml to 1380 ug/ml. Please double check it.

Reply #2: *The reviewer is correct in pointing out that we did use these high concentrations of Revactin® in our cell culture experiments. The reason for this is that we wanted to mimic the in vivo concentration used in our earlier human studies. As we now show in our new figure 2, at 50% of the concentration used in our human in vivo studies, there was an increase in nitrite formation but not an increase in cGMP levels suggesting that the amount of NO produced at 50% of the normal in vivo dose of Revactin® is insufficient to stimulate the formation of cGMP.*

Changes in the text: We added a sentence clarifying the use of high concentrations of Revactin® in the material and methods section, page 8, lines 9-11.

Comment 3. How many Primary human corpora cavernosa cells were used this experiment? Please provide the “n” for statistical analysis.

Reply 3: In our experiments, we used 6-well plates. Cells are seeded at 0.2×10^6 cells until they reached confluency at 1.0×10^6 cells to start the treatments. We have done the experiments using 4 different human donors. We have used $n=4$ for all the statistical calculations. Each experiment was repeated at least thrice from the 4 donors.

Changes in the text: We have added the number of cells seeded in all the experiments in the materials and methods sections, page 7, lines 19-23, and page 12, lines 9-12.

Comment 4. There are several typos on human cavernosal smooth muscle cells (HCSMC). In the figure legend, the author used HCSM. Please correct them.

Reply 4: It has now been corrected throughout the manuscript. Only remains as CSMC when referring to rat cavernosal smooth muscle cells.

Changes in the text: HCSM was changed to HCSMC throughout the entire manuscript.

Comment 5. Please provide images at high resolution >300dpi.

Reply 5: We have added new figures at high resolution for figure 1 at 600 dpi.

Reviewer B

In the present study Ferrini et al. aimed to determine whether a commercial formulation of COMP-4 (combination of ginger, Paullinia cupana, muira puama and L-citrulline), Revactin[®], could stimulate iNOS (inducible nitric oxide synthase), nitric oxide (NO) and cGMP in human cavernosal smooth muscle cells (CSMC). With this purpose, in vitro assays were carried out in primary human CSMC cultures. It was concluded that Revactin[®] was capable of activating the iNOS-NO-cGMP pathway intracellularly within the human CSMC. I have the following comments:

Comment 1. Authors have previously shown similar results in rats (PMID: 29551532; PMID: 26405615). Thus, it is not clear what is (are) the novelty of the present study. I mean, it will be expected that COMP-4 would activate the nitrenergic pathway in the human CSMC (as it did in rats). The results presented here should have been published with those previous observations in rats.

Reply 1: The reason for conducting these experiments on the HCSMC was to corroborate whether Revactin[®], a slightly different formulation of COMP-4, was also capable of activating the human iNOS- NO-cGMP pathway. When our rat paper was published in 2018, we did not have any HCSMC data at that time.

Changes in the text: none

Comment 2. It has been previously demonstrated by the authors that COMP-4 displayed antioxidant effects and no changes in nitrotyrosine levels were detected after in vitro treatment with COMP-4 (PMID: 32005937). This finding seems contradictory considering the present results showing that COMP-4 induced iNOS expression. It is well established in the literature that iNOS-derived NO is responsible for protein nitration (via ONOO-).

Reply 2: The reviewer is correct in that NO derived from iNOS is responsible for protein nitration via peroxynitrite, ONOO-. However, in addition to this increase in NO from iNOS, there must be a concomitant increase in reactive oxygen species. As the reviewer has pointed out [PMID: 32005937], we have previously demonstrated that COMP-4 has antioxidant effects, thereby reducing oxidative stress and peroxynitrites formation.

The in vitro results presented in this manuscript do not appear to contradict our previous in vivo findings in the aged rat corpora cavernosa. In those aged rat corpora cavernosa, COMP-4 increased iNOS expression, but it did not result in any changes in the nitrotyrosine levels. As we point out in the present discussion, we believe that the anti-oxidative effects of Revactin® would result in a decrease in reactive oxygen species making NO more bioavailable for other cellular and physiological functions.

Changes in the text: we have added this text in the discussion section page 16, lines 18-22.

Comment 3. It is not clear whether induction of iNOS may be considered a beneficial effect since iNOS is considered a pro-inflammatory protein. In this sense, induction of iNOS is usually associated with cell damage. iNOS produces large amounts of NO, which may react with ROS to produce ONOO, a powerful oxidant molecule. Therefore, it should be interesting to check for lipoperoxidation and nitrotyrosine in cells incubated with Revactin®.

Reply 3 The dogma that eNOS and nNOS are important regulators of many physiological processes, whereas iNOS is only associated with inflammation, oxidative stress, and cell death is undergoing reassessment. It is important to note that not all studies support a detrimental role for iNOS. It has been observed in bone that iNOS (expression) is fundamental for initiating bone healing process after a fracture (1), and iNOS has been implicated in the process of repairing the intestinal mucosa after chronic injury (2,3). In addition, it has been shown that delivery of iNOS to skin using adenoviral vectors improved healing of this tissue in iNOS-/- mice, suggesting that iNOS may be therapeutically useful if given in appropriately regulated amounts at specific sites (4).

Our future directions regarding the effect of Revactin® will center on the expression of nitrotyrosine and lipid peroxidation after inducing oxidative stress. For the current manuscript, we restricted our investigation to the effect of Revactin® on the NO-cGMP pathway within the HCSMC.

1. Diwan AD, Wang MX, Jang D, Zhu W, Murrell GA. Nitric oxide modulates fracture healing. *J Bone Miner Res.* 2000 Feb;15(2):342-51. doi: 10.1359/jbmr.2000.15.2.342. PMID: 10703937.
2. McCaVerty D, Mudgett JS, Swain MG, Kubes P. Inducible nitric oxide synthase plays a critical role in resolving intestinal inflammation. *Gastroenterology* 1997;112:1022-7.
3. Kubes P Inducible nitric oxide synthase: a little bit of good in all of us. *Gut* 2000;47:6-9

4. Yamasaki K, Edington HD, McClosky C, *et al*. Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J Clin Invest* 1998;**101**:967–71.

Changes in the text: New references have been added as references 29, 31-33 discussing the beneficial effect of iNOS. Discussion section, page 17 lines 1-11.

Comment 4. Page 4: Please clarify how the concentrations of Revactin[®], IBMX and L-NIL and were chosen. It is not clear how the period of incubation was determined. It is important to show a curve for period of incubation with the tested drugs.

Reply 4: The concentration of L-NIL and IBMX were selected by searching the literature and by our own experience in working with these two compounds. For choosing the concentration of Revactin[®], we converted the dose employed in humans to a cell culture concentration that would not affect cell viability. We used 50%, 100%, and 200% of the in vivo human dose.

As per the reviewer request, we have included the time course of cGMP production with 100% Revactin[®] and sildenafil as a positive control. We selected 100% Revactin[®] because it was the concentration that gave us the maximum output of cGMP in our experiments.

Changes in the text #4

As such, we have added a new figure, Figure 3, showing the time course of Revactin[®] in HCSMC. The description of the results is now included in the results section, page 13, lines 17-22. The time course protocol is now included in the materials and methods section, page 9, lines 19-24.

Comment 5. Page 7: The method used to determine nitrite concentration is not appropriated. Considering that evaluate NO levels is one of the most important objectives of the study, this experiment should be carried out using a more sensible technique.

Reply 5: The Griess reaction for the determination of nitrite used in our experiments has been extensively utilized in over 2,000 publications in the literature. We do agree that there are more sensitive methods for measuring NO; but in our experiments we are observing changes in the formation of nitrite in the absence or presence of Revactin[®] or L-NIL. We do not believe that using a more sensitive method than the Griess reaction would add anything different from the results already observed in our experiments.

Changes in the text#5: none

Comment 6. Figure 4: iNOS is an inducible protein. How authors explain the high expression of this protein in cells not stimulated with Revactin[®] (representative Fig. 4A). Note that eNOS and nNOS have a lesser expression (Fig. 4B and C).

Reply 6: We have been working with iNOS expression in smooth muscle cells in the penis and peripheral vasculature for more than 10 years and have shown in both tissues that it increases with aging. We have always found a low expression of iNOS in untreated smooth muscle cells not only by WB but also by immunohistochemistry. Here are several papers that show endogenous expression of iNOS not only in smooth muscle cells but in brain, bone, and gut (ref

29,30,31).

Changes in the text: References 28-32 has been added to the discussion.

Comment 7. Figures 2 and 3 should be merged. The same apply to Figs. 5 and 6.

Reply 7: As the reviewer has requested we have merged figures 2 and 3; they are now figure 2 A and 2B; the same applies to figures 5 and 6 which are now figure 5A and 5B

Changes in the text: Figure numbers have been changed in the results section page 13, lines 1 and 9 for figure 2; page 14, lines 10 and 11 for figure 5, and in the legends of the figure

Comment 8. Fig. 6.: Inhibition of iNOS with L-NIL decreased the basal levels of cGMP suggesting that iNOS contributes to the basal production of NO in CSMC. How is that possible. The constitutive isoforms of NOS are eNOS and nNOS and these are the main enzymes producing NO in these cells.

Reply 8: the reviewer is correct in stating that the two constitutive isoforms of NOS, nNOS and eNOS, have been shown to produce NO (in the penis) but not specifically in the cells we are studying in these experiments which are the HCSMC. There is universal agreement that NO from nNOS is synthesized and then released by the cavernosal nerve terminals into the CSMC but it does not emanate from the CSMC themselves. Regardless of whether one agrees or not with the experimental data in the literature that proposes that the sinusoidal endothelium makes NO from eNOS, this isoform if it is involved in the erectile response also does not reside within the CSMC themselves. While iNOS within the cells of the tunica albuginea (TA) has been shown to be active in making NO when there is an injury to the TA, it is now recognized that with aging the iNOS isoform becomes upregulated within the CSMC and the NO from this upregulated iNOS within the CSMC acts presumably as an anti-fibrotic and anti-apoptotic molecule against the oxidative stress of aging that is ongoing within these aging CSMC. So it is not surprising to see a basal level of active iNOS activity within the CSMC as is evidenced by the drop in intracellular levels of cGMP and nitrite with L-NIL, the specific inhibitor of L-NIL.

Changes in the text #8: we have added this text to the discussion page 17, lines 12-19.

9. It is important to show how Revactin[®] induced iNOS expression.

Reply 9: Since we have shown in both rat (19) and human corpora smooth muscle cells that iNOS protein expression can be increased by COMP-4 and Revactin[®], respectively, it is possible that Revactin[®] could be modulating mRNA expression or promoting post-transcriptional modifications that would increase the synthesis of the iNOS protein.

Changes in the text: We have added this to the discussion section page 15 lines 4-8.